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Coimmobilization of a redox enzyme and a cofactor regeneration system†

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The coimmobilization of nitrobenzene nitroreductase and glucose-6-phosphate dehydrogenase in silica particles enables the continuous conversion of nitrobenzene to hydroxylaminobenzene with NADPH recycling.

Cofactor dependent oxidoreductases catalyze a wide range of enantio- and regio-selective reactions.¹ In intact cells, redox cofactors such as NADPH are continuously regenerated by cellular metabolism. Therefore, whole-cell biocatalysis is widely used for redox reactions including asymmetric hydroxylations and epoxidations.^{2a} Unfortunately, whole-cell systems are often limited by product toxicity, byproduct formation, poor substrate uptake rates and difficulty with product recovery following catalysis.^{2b} The widespread use of purified redox enzymes in biocatalysis is limited by the cost of supplying stoichiometric amounts of cofactors for catalysis. An increasing interest in preparative pure enzyme applications necessitates a search for efficient and robust strategies for *in situ* cofactor recycling.^{2c} Several cofactor regeneration systems have been demonstrated using dehydrogenase enzymes.³ Current systems for NADPH regeneration, however, have not been successfully applied to large scale synthesis, primarily due to the low total turnover number of the recycling enzymes.^{2b} A high specific activity and a strategy for removal of the enzymes during product recovery are goals of an efficient recycling system, but the stability of the enzyme under reaction conditions must be optimized.

The NADPH-dependent nitrobenzene nitroreductase (NBNR) from *Pseudomonas pseudoalcaligenes* JS45 catalyzes a four-electron reduction of nitrobenzene to hydroxylaminobenzene (HAB) and has been successfully employed for whole-cell biocatalysis in *o*-aminophenol synthesis.⁴ However, intact cells have a relatively low specific activity and both substrate and product can be toxic to the cells.^{4b} The applicability of the purified enzymes as biocatalysts is also limited by the requirement for two moles of NADPH per mole of substrate.

The encapsulation of enzymes in silica nanoparticles imparts exceptional stability and high loading capacities for the resulting biocatalysts.⁵ We recently reported the preparation of immobilized-NBNR by encapsulation within silica particles using

polyethyleneimine (PEI) to direct silica formation.⁶ Immobilized NBNR provides a stable and reusable catalyst, but is still limited by a requirement for a stoichiometric amount of NADPH.^{5c} The high loading capacity and versatility of the immobilization system however, led us to investigate the potential for coimmobilization of two enzymes working in tandem. The immobilization of sequentially acting enzymes within a confined space increases the catalytic efficiency of the conversion due to a dramatic reduction in the diffusion time of the substrate. Moreover, the *in situ* formation of substrates generates high local concentrations that lead to kinetic enhancements that can equate to substantial cost savings.⁷ We report here the coimmobilization of NBNR and glucose-6-phosphate dehydrogenase (G6PDH) in a multi-enzyme system for the continuous reduction of nitroaromatic compounds. In this model system NADP⁺ dependent-G6PDH catalyzes the recycling of NADPH *in situ* providing a constant source of reducing equivalents to NBNR for the reduction of nitrobenzene to HAB (Fig. 1A).

NBNR and G6PDH were efficiently coimmobilized in PEI-directed silica particles with negligible loss in activity (Table 1). Kinetic analysis of G6PDH and NBNR activities in the coimmobilized suspension revealed that the apparent K_m of the PEI silica-encapsulated G6PDH for exogenously added NADP⁺ was comparable to that of the soluble enzyme (150.8 ± 12.5 and $156.5 \pm 8.8 \mu\text{M}$ respectively). The apparent K_m value of PEI silica-encapsulated NBNR for exogenously added NADPH was about 3 times higher than that of the soluble enzyme (344.6 ± 28.3 and $116.5 \pm 11.7 \mu\text{M}$ respectively). Modifications of K_m values for immobilized enzyme preparations can be attributed to substrate diffusion limitations and steric hindrances compared to the soluble forms.⁸ The kinetic measurements above were all made with exogenously added cofactors. The actual K_m for NADPH recycled *in situ* may be substantially lower because the NADPH is formed near the site of the reaction and therefore much less limited by diffusion. Despite the differences in the kinetic parameters of the immobilized and soluble enzymes, the dramatic operational and thermal stabilization achieved by silica precipitation⁶ would balance any negative effect of these parameters on the reaction.

The amount of G6PDH required to achieve maximum HAB formation was optimized by adjusting the ratio of NBNR : G6PDH in the coimmobilized preparation. The conversion of nitrobenzene to HAB requires two molecules of NADPH for each molecule of nitrobenzene, indicating a theoretical optimum ratio of enzyme units (U) of 1 U NBNR : 2 U G6PDH. Maximal nitrobenzene conversion was achieved however, by using an excess of G6PDH, with an optimum of 1 U NBNR : 5 U G6PDH. With a starting concentration of 100 μM nitrobenzene, the initial activity

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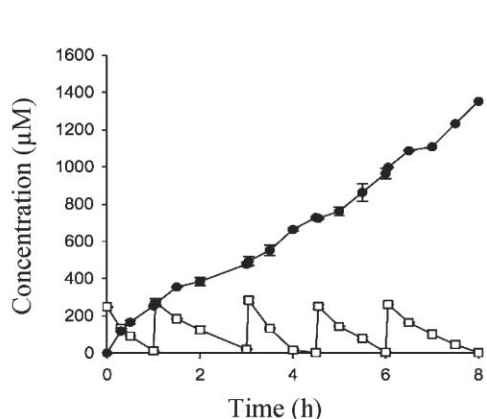
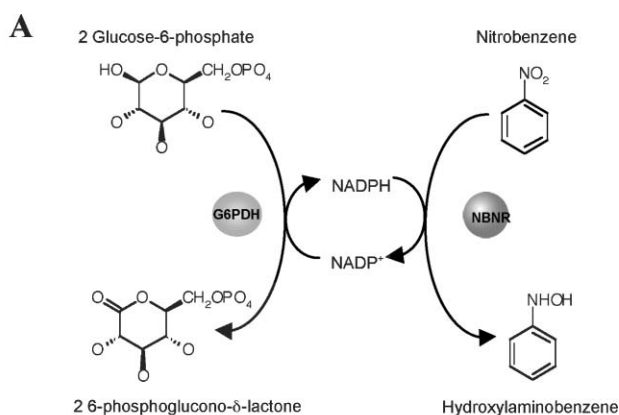


Fig. 1 A. Schematic and B. observations of the continuous conversion of nitrobenzene (100 μM) to HAB and NADPH recycling by a 1 U NBNR : 5 U G6PDH system. Nitrobenzene (\square), HAB (\bullet), 240 μM NADPH and 3 mM glucose-6-phosphate added at 0 h. An additional 3 mM glucose-6-phosphate was added after 3 h.

Table 1 Immobilization data

Enzyme preparation	Immobilization yield (%) ^a	Immobilized activity yield (%) ^b
NBNR	99.5 (± 0.7)	58.7 (± 4.6)
G6PDH	99.6 (± 0.6)	33.3 (± 0.4)
Coimmobilized NBNR	100 (± 0)	52.5 (± 0.2)
Coimmobilized G6PDH	98.4 (± 0.6)	33.0 (± 0.3)

^a Yield (%) = (initial activity – activity in the supernatant) \times 100/initial activity. ^b Immobilized activity yield (%) = measured activity in the immobilized enzyme \times 100/(initial activity – activity in the supernatant).

of coimmobilized NBNR–G6PDH increased linearly with increasing NADPH concentrations up to 240 μM . The optimized formulation (1 U NBNR : 5 U G6PDH and 240 μM NADPH) was used to test the recycling system in the continuous conversion of nitrobenzene (Fig. 1B). Nitrobenzene was supplied to the system in 250 μM increments to a total of 1.5 mM. NADPH was supplied to initiate the reaction and coupling of NBNR and G6PDH activities was evidenced by the continuous formation of HAB for 8 h without further addition of the cofactor. The conversion efficiency of the reaction was 90% with a final yield of 1.35 mM HAB. The conversion of nitrobenzene to HAB over an 8 h period of sustained activity was reproducible for initial concentrations of nitrobenzene up to 10 mM with no loss in the capacity to

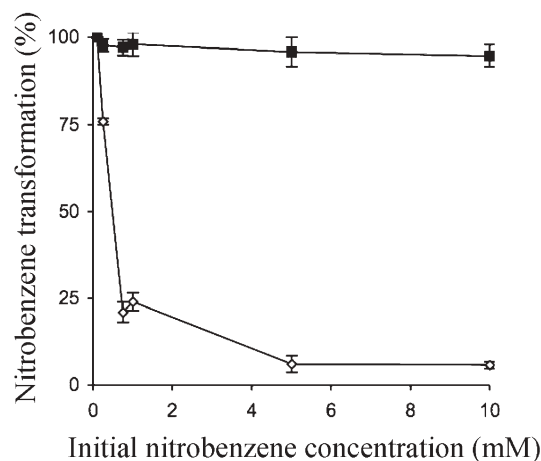


Fig. 2 Transformation of nitrobenzene. Reaction time: 8 h, 240 μM NADPH. 1 U NBNR : 0 U G6PDH (\diamond), 1 U NBNR : 5 U G6PDH (\blacksquare).

transform nitrobenzene (Fig. 2). Control reactions containing immobilized-NBNR alone lost activity rapidly as the supply of NADPH became exhausted, leading to incomplete conversion of nitrobenzene (Fig. 2). Control reactions containing immobilized-G6PDH alone did not transform nitrobenzene (data not shown). When nitrobenzene was added in 10 mM increments, transformation was reproducible up to 30 mM (Fig. 3, ESI†). Estimation of HAB concentration became problematic for concentrations higher than 15 mM, probably due to instability or precipitation of the product. Activity of the biocatalyst, however, was undiminished after conversion of 30 mM nitrobenzene as shown by the full recovery of the enzyme activity after washing the coimmobilized system by centrifugation (Fig. 4, ESI†).

The use of immobilized enzymes for cofactor regeneration is receiving increasing attention,⁹ however, there are few literature reports demonstrating coimmobilization as a strategy for biocatalysis with cofactor recycling systems¹⁰ and such studies typically do not report a total turn-over number (moles of product formed/moles of cofactor present in the reaction) for NADPH. The system described herein significantly enhanced product formation (up to 125 fold with respect to the non coupled system) with a total turn-over number for NADPH of 62 under the tested conditions. We did not attempt to optimize the system to maximize the total turnover number but it is clear that our estimate is conservative.

Enzyme entrapment in silica allows the preparation of active and stable composites. Coimmobilizing a catalytic enzyme with a cofactor-regenerating enzyme provides a variety of potential advantages including: continuous operation, catalyst reuse, cost reduction and simplified product isolation. The mild immobilization reaction is widely applicable to a range of biomolecules for application to a variety of potentially interchangeable multienzyme configurations.

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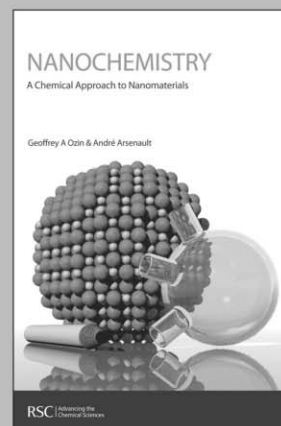
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